Organogenesis 10:1, 108–125; January/February/March 2014; © 2014 Landes Bioscience

Cilia and coordination of signaling networks during heart development

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Keywords: nodal cilia, primary cilia, signaling networks, cardiomyogenesis, stem cells, heart development, heterotaxy, congenital heart diseases

Abbreviations: Ablim1, actin binding lim protein 1; AoS, aortic stenosis; ALMS, Alström syndrome; ASD, atrial septal defect; AV, atrioventricular; AVC, atrioventricular canal; AVCD, atrioventricular canal defect; AVS, aortic valve stenosis; AVSD, atrioventricular septal defect; BBS, Bardet-Biedl syndrome; BMP, Bone Morphogenic Protein; CA, common atrium; CCV, clathrin coated vesicle; CDE, clathrin dependent endocytosis; CHD, congenital heart disease; DWS, Dandy-Walker syndrome; CoA, coarctation of the aorta; DORV, double outlet right ventricle; Dvl, Dishevelled; E, embryonic day; EE, early endosome; EGFR, Epidermal Growth Factor Receptor; EMT, epithelial-to-mesenchymal transformation; EndoMT, endothelial-to-mesenchymal transformation; EVC, Ellis-van Creveld syndrome; FGFR, Fibroblast Growth Factor Receptor; FHF, first heart field; Ftm, Fantom; Fz, Frizzled; GPCRs, G-protein coupled receptors; hESC, human embryonic stem cells; Hh, Hedgehog; HLHS, hypoplastic left heart syndrome; IDA, inner dynein arm; IFT, intraflagellar transport; JBTS, Joubert syndrome; L-R, left-right; LRD, left-right dynein; MAPK, Mitogen Activated Protein Kinase; MKKS, McKusick-Kaufman syndrome; MKS, Meckel syndrome; NPHP, nephronophthisis; NVP, nodal vesicular parcels; ODA, outer dynein arm; OFD, Orofaciodigital syndrome; OFT, outflow tract; PC, polycystin; PCP, planar cell polarity; PDA, patent ductus arteriosus; PDGFR, Platelet Derived Growth Factor Receptor; PDGFRα, Platelet Derived Growth Factor Receptor alpha; PDGFRβ, Platelet Derived Growth Factor Receptor beta; Pifo, Pitchfork; PLSVC, persistent left superior vena cava; PS, pulmonary stenosis; PTA, persistent truncus arteriosus; Ptch, Patched; RTK, Receptor Tyrosine Kinase; SHF, second heart field; Shh, Sonic hedgehog; SI, Situs Inversus; Smo, Smoothened; SRPS, Short-rib polydactyly syndrome; SS, Sensenbrenner syndrome; SV, single ventricle; TGA, transposition of the great arteries; TGFβ, Transforming Growth Factor beta; TOF, tetralogy of Fallot; VSD, ventricular septal defect; Wnt, Wingless-type Integration site; wt, wildtype

Primary cilia are unique sensory organelles that coordinate a wide variety of different signaling pathways to control cellular processes during development and in tissue homeostasis. Defects in function or assembly of these antenna-like structures are therefore associated with a broad range of developmental disorders and diseases called ciliopathies. Recent studies have indicated a major role of different populations of cilia, including nodal and cardiac primary cilia, in coordinating heart development, and defects in these cilia are associated with congenital heart disease. Here, we present an overview of the role of nodal and cardiac primary cilia in heart development.

Introduction

The heart is the first organ to form in the developing embryo to ensure distribution of nutrients and oxygen during fetal development.[1] Cardiogenesis is a complex process of highly coordinated events that include heart tube formation and looping, chamber septation, and maturation. The complexity of heart development is reflected by the high occurrence of congenital heart disease (CHD), which appear in almost 1% of all live births, thereby comprising the most common congenital disorder. Despite increasing knowledge and therapeutic advances, CHD causes 10% of all noninfectious infant deaths within the first year of life.[2,3] The spectrum of CHD is broad and patients may have more than one heart abnormality. CHD can be characterized by multifaceted morphological and structural abnormalities including septal and valve defects, tetralogy of Fallot (TOF) and arterial transposition.[4,5] CHD prognosis and mortality depend on the size, number, and type of defect(s) and the associated abnormalities.[2] An overview of common types of heart defects and their morphological characteristics is given in **Table 1**.

Throughout heart development, different types of cilia are expressed in a spatiotemporal manner to control various aspects of cardiogenesis. During gastrulation, motile and sensory cilia at the embryonic node (**Fig. 1A**) play a critical role in regulating signaling processes required for the establishment of left-right (L-R) organ asymmetry, a process which controls the initial stages of heart morphogenesis and connections to the vasculature.[6-10] Consequently, defects in L-R signaling result in a variety of heart defects that arise from abnormal looping and remodeling of the primitive heart tube into a multi-chambered

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Submitted: 10/01/2013; Revised: 12/06/2013; Accepted: 12/10/2013;
Published Online: 12/17/2013; http://dx.doi.org/10.4161/org.27483

Table 1. Common types of congenital heart defects

Congenital heart defect	Abbreviation	Characteristics
Aortic stenosis	AoS	Obstruction of blood flow between left ventricle and the aorta. This may be caused by abnormalities of the aortic valve (aortic valve stenosis, AVS), muscular obstruction or narrowing of the aorta immediately above the valve.
Atrial septal defect	ASD	Incomplete septation of the atria.
Atrioventricular septal defect	AVSD	Developmental defects that arise from developmental defects of the endocardial cushions. Such defects affect may affect the lower part of the atrial septum, the ventricular septum and the mitral and tricuspid valves.
Coarctation of the aorta	CoA	A narrowing of the aorta.
Hypoplastic left heart syndrome	HLHS	All structures of the left side of the heart, including the left ventricle, mitral and aortic valves, are severely underdeveloped.
Patent ductus arteriosus	PDA	Failure of closure of the ductus arteriosus (DA) at birth. DA is a blood vessel which allows passage between the pulmonary artery and the aorta. This passage allows bypass of the lungs in fetal circulation.
Persistent left superior vena cava	PLSVC	Failure of obliteration of the left superior vein.
Transposition of the great arteries	TGA	The aorta and pulmonary artery are reversed, so that the aorta arises from the right ventricle and the pulmonary artery arises from the left ventricle. The result is that there is no connection between systemic and pulmonary circulation.
Tetralogy of Fallot	TOF	Involves four anatomical abnormalities in the heart: 1) Ventricular septal defect (hole between ventricles) 2) Pulmonary stenosis (pulmonary artery is narrow) 3) Overriding aorta (the aorta is positioned between the two ventricles) 4) Hypertrophic (thickening of) right ventricle.
Ventricular septal defect	VSD	Incomplete septation of the ventricles.

Information from Cincinnati Childrens Hospital. Heart Institute Encyclopedia. http://www.cincinnatichildrens.org and American Heart Association. About congenital heart diseases. www.heart.org.

organ.[11-19] In addition, primary cilia that are present in cardiomyocytes and within the developing heart, also known as cardiac primary cilia (**Fig. 1B**), are compartmentalized with a series of receptor systems,[20-22] which take part in regulating cellular signaling pathways important for the progressive differentiation, morphogenesis and maturation of the heart. This suggests that also cardiac primary cilia play a role in coordinating the signaling networks that are required for proper heart development. Here we present an overview of cilia, ciliopathies and heart development with focus on recent advances in understanding the role of different populations of cilia in coordinating signaling networks during heart development, and we discuss how defects in ciliary formation, motility, and sensory reception may lead to CHD.

Cilia, Ciliopathies and CHD—An Overview

Cilia are membrane-bound, microtubule-based organelles that play important roles in motility and sensation. Cilia project from the surface of most animal cells, from protists to humans.[23] In vertebrates, cilia are generally divided into two types according to their axonemal arrangement and ability to engage active movement. Axonemes in multiciliated cells of mammalian epithelia (e.g., in brain ventricles, oviduct, and airways) usually have a 9+2 composition of microtubules, possess dynein arms, radial spokes, and are motile. Ciliary motility is regulated by outer (ODA) and inner (IDA) dynein arms that control ciliary beat frequency and form, respectively, and power the movement of fluid or substances over an epithelium. In contrast, non-motile primary cilia, which are found in a single copy on the surface of most quiescent cells in the body, usually have a 9+0 microtubule composition and lack dynein arms and radial spokes.[23] Both motile and primary cilia are subtended by a modified centriole called the basal body, which for primary cilia corresponds to the most mature (mother) centriole of the centrosome (**Fig. 1B**), and rely on intraflagellar transport (IFT) for their assembly, length, maintenance, and signaling properties. IFT is a specialized bidirectional trafficking system with motor molecules, IFT complexes, and other adaptor proteins that move axonemal precursors (e.g., tubulin) and certain ciliary membrane proteins into and out of the cilium.[24] Canonical anterograde transport of proteins from the ciliary base to the tip is mediated by the heterotrimeric kinesin-2 motor protein, comprising Kif3a, Kif3b and Kap, whereas retrograde IFT is mediated by cytoplasmic dynein-2.[25,26]

Primary cilia play fundamental roles as chemo- and mechanosensory organelles and coordinate numerous signaling pathways, including the Hedgehog (Hh), Wingless-type Integration site (Wnt), Receptor Tyrosine Kinase (RTK), Notch, and Transforming Growth Factor β (TGFβ) signaling systems, as well as signaling through receptors for extracellular matrix proteins, ion channels, and a wide variety of different G-protein coupled receptors (GPCRs). By these means, primary cilia control cellular processes during development and in the adult organism.[20,27-36] In addition, specialized non-motile sensory cilia are present on neurons in the olfactory epithelium of the nasal

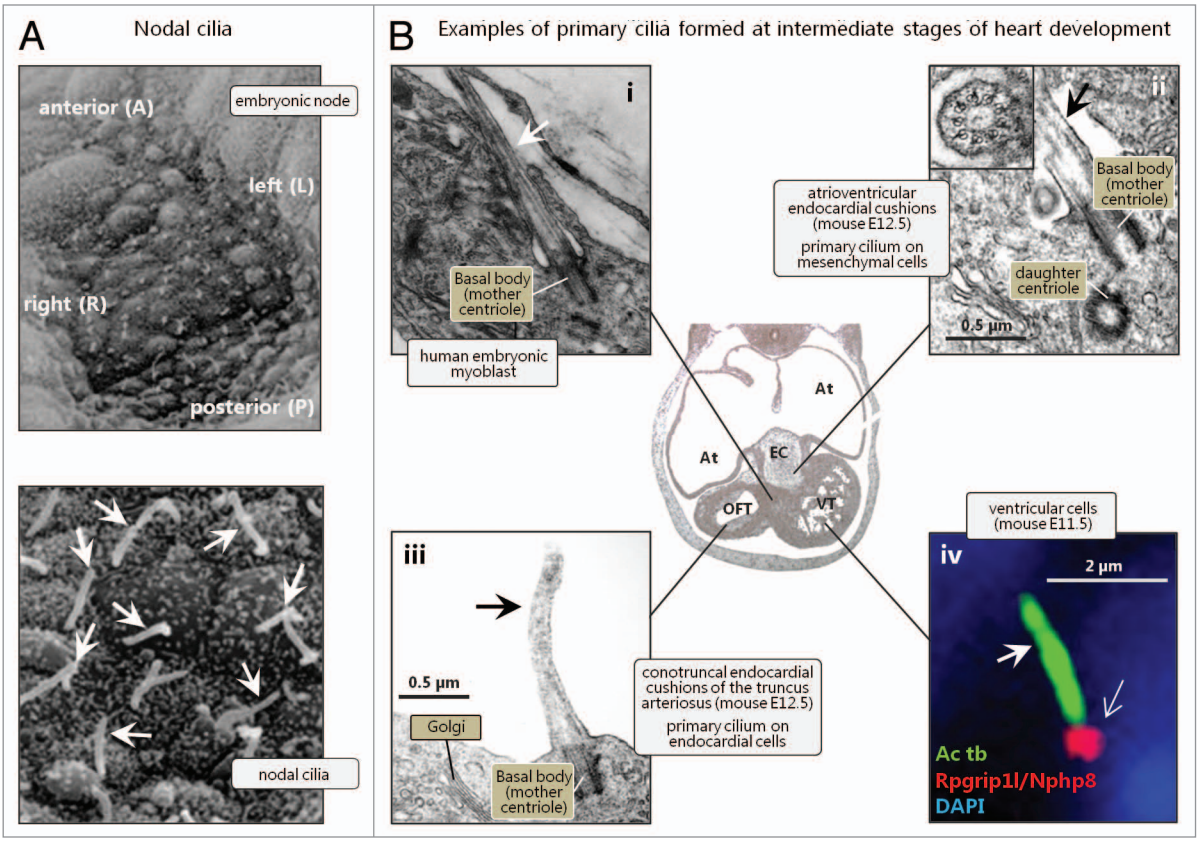

Figure 1. Different populations of cilia in the developing heart. (**A**) Scanning electron microscopy images of nodal cilia (arrows) at the embryonic node. Reproduced from ref. 8 with permission. (**B**) Transmission electron microscopy (**i, ii, and iii**) and immunofluorescence microscopy (IFM) (**iv**) images of cardiac primary cilia (arrows) emanating from the centrosomal mother centriole that functions as a basal body. In the IFM analysis, the primary cilium was marked with an antibody against acetylated α-tubulin (Ac tb; *green*), and the lower part of the cilium (open arrow) was marked with an antibody against Nephrocystin 8 (Rpgrip1l/Nphp8; *red*). Nuclei were marked with DAPI, which stains DNA (*blue*). Abbreviations: At: Atrium; EC: endocardial cushions; OFT: outflow tract; VT: ventricle. Reproduced from ref. 133 (**i**), 142 (**ii and iii**), and 22 (**iv**) with permissions.

cavity and on photoreceptor cells.[37,38] Lastly, motile (nodal) cilia with ODA, also known as Left-Right dynein (LRD), are found during gastrulation at the embryonic node (**Fig. 1A**) and play a decisive role in laterality establishment.[6,39] These cilia were originally reported to display 9+0 axonemes,[8] but nodal cilia with a 9+2 and even 9+4 axonemal structures have also been found in mouse, rabbit,[40,41] and zebrafish.[42] Thus, motile as well as non-motile cilia with variable architecture of axonemal microtubules are present in multiple tissues and organs throughout the body where they regulate key events during development and in the adult. Therefore, defects in genes required for ciliary assembly, maintenance, motility, and/or sensory functions may lead to a series of syndromic diseases and developmental disorders referred to as ciliopathies. The clinical features of ciliopathies include laterality defects, airway dysfunction, sterility, cognitive disorders, skeletal bone, renal, hepatic, pancreatic, and brain defects, retinal degeneration, anosmia, obesity, and cancer.[43-46]

The multifaceted diseases and developmental defects associated with dysfunctional cilia reflect the complexity and importance of these organelles throughout life. Most importantly, defective cilia are also associated with CHD. Heart defects are observed in several ciliopathy syndromes, including Alström syndrome (ALMS), Bardet-Biedl syndrome (BBS), Meckel syndrome (MKS), Dandy-Walker syndrome (DWS), Joubert syndrome (JBTS), Ellis-van Creveld syndrome (EVC), McKusick-Kaufman syndrome (MKKS), Short-rib polydactyly syndromes (SRPS), Sensenbrenner syndrome (SS), and nephronophthisis (NPHP) (**Table 2**). In many cases, ciliopathy-associated CHDs display the typical heart defects observed in patients with laterality defects, yet several other types of CHD have been reported in ciliopathy patients, including septum defects, and aortic stenosis.[47-51] As an example, NPHP proteins, the nephrocystins, critically regulate the recruitment and access of proteins to the cilium, such as through the formation of ciliary gating complexes.[52-61] In this regard, NPHP2/inversin recruits and anchors NPHP3, NPHP9/Nek8, and the newly identified NPHP protein, Anks6, to a distinct region in the proximal cilium, the inversin compartment, and integrity of this module is essential for correct laterality establishment in organisms from zebrafish to humans.[15,62-68] Inversin was first discovered for its role in L-R establishment[62] and, presumably, the inversin compartment impinges on the function of motile and/or sensory cilia at the embryonic node,[67,69] which is critical in breaking the embryonic bilateral symmetry through the generation of a net

Table 2. Human ciliopathies that include cardiac phenotypes

Ciliopathy	Abbreviation	OMIM number	Heart defects observed in patients [a]
Alström syndrome	ALMS	203800	Dilated cardiomyopahty in 60% of cases[217]
Bardet-Biedl syndrome	BBS	209900	CHD in 7–18% of cases. Types of CHD include ASD,PDA VSD, AS, aortic valve stenosis, CoA, atrioventricular canal defect(AVCD), subvalvular stenosis, cardiomyopathy[50,218-220]
Dandy-Walker syndrome	DWS	220200	CHD in 36% of cases. VSD, HLHS, hypoplastic right heart, ASD, TOF[221]
Ellis-van Creveld syndrome	EVC	225500	CHD in 60% of cases. Types of CHD often include AVCD, Common atrium and PLSVC, but other defects are also observed[47,49,222]
Joubert syndrome and related disorders	JBTS	213300	Heart defects include AoS, ASD, dicuspid aortic valve, PLSVC, TGA[47,48,223]
McKusick-Kaufman syndrome	MKKS	236700	CHD in 14% of cases. Types of CHD include AVCD, PLSVC, TGA, HLHS, VSD, ASD as well as other defects.[47,50]
Meckel syndrome	MKS	249000	CHD in 16% of cases. Types of CHD include ASD, VSD, bicuspid aortic valve and HLHS[224]
Nephronophthisis	NPHP1, 2, 3, 4 and 11	256100, 602088, 604387, 606966, 613550	Frequency of CHD is very variable and perhaps dependent on disease gene. Cardiac phenotypes include Cardiomyopathy, SI, dextrocardia, TGA, AVCD, DORV, VSD, ASD and AS[13,14,68,225]
Orofaciodigital syndrome, type II and type IV	OFD II, OFD IV	252100, 258860	Heart defects include AVCD, CA, HLHS, CoA[47]
Sensenbrenner syndrome	SS	218330	Bicuspid aortic valve. ASD hypertrophic left ventricle[226]
Short rib-polydactyly syndrome I-IV	SRPS I-IV	263530, 263520, 615087, 263510, 269860	Heart defects include AVCD, PLSVC, TGA, HLHS, CoA, VSD, ASD[47]

[a]AS, aortic stenosis; ASD, atrial septal defect; AVCD, atrioventricular canal defect; AVS, aortic valve stenosis; CA, common atrium; CoA, coarctation of the aorta; DORV, double outlet right ventricle; HLHS, hypoplastic left heart syndrome; PDA, Patent ductus arteriosus; PLSVC, persistent left superior vena cava; TGA, transposition of the great arteries; TOF, tetralogy of Fallot; VSD, ventricular septal defect.

leftward flow of the embryonic fluid during gastrulation[8] (see also below). Consequently, NPHP patients occasionally present with laterality defects and associated complex CHD.[13,15,47,66,68,70-72] At later stages of heart development, primary cilia may assist in coordinating signaling networks required for morphogenesis and maturation of the heart.

Major Steps in Heart Development

Two different cell populations of mesodermal origin, i.e., the first (FHF) and second (SHF) heart fields, participate in mammalian heart development in a spatiotemporal manner; cells from FHF contribute to the ventricles, the atria and the atrioventricular canal (AVC), whereas cells from SHF contribute to the outflow tract (OFT) and all other regions of the heart, except for the left ventricle (**Fig. 2**).[73-75] At embryonic day (E) 6.5 in mice, myocardial progenitor cells undergo epithelial-to-mesenchymal transformation (EMT) and ingress through the primitive streak while giving rise to the mesodermal layer forming the FHF. On each side of the midline, the FHF resides as two patches of mesodermal cells that extend across the midline and at E7.5, they fuse to form a crescent-shaped epithelium called the cardiac crescent. Cells from SHF initially lie medially to FHF precursor cells at E7.0–7.5.[73] During embryonic folding at E8 in mice and day 21 in humans, the cardiac crescent fuses along the midline forming the early heart tube (reviewed in refs. 1 and 76).

To form the cardiac chambers, the heart tube loops and expands regionally. Parts of the heart tube, which form the outer curvature of the looped heart tube, begin to gain a chamber myocardium gene expression profile, and the tissue expands in a balloon-like fashion to eventually become the atrial and ventricular chambers.[77] Specific parts of the heart tube retain a non-chamber myocardium gene expression profile, and function as "rings" of non-expanding myocardium, which participate in formation of the four-chambered heart.[78] This non-expanding myocardium consists of the inflow tract, the AVC and the OFT. Reciprocal signaling in the AVC and OFT between the endocardium and the myocardium induces endocardial cells to undergo endothelial to mesenchymal transformation (EndoMT), and invade the extracellular matrix, also known as the cardiac jelly, to form the endocardial cushions which will eventually be transformed into cardiac valves and participate in septation of the OFT.[79] Cells from the SHF and the neural crest also participate in septation of the OFT.[75,80] The left and right atrium and ventricle become separated by muscular partitions, which grow from the atrial roof and the ventricular floor and meet endocardial cushions of the AVC and OFT.[81]

Heart Development is Coordinated by Multiple Signaling Networks

Heart development is regulated by a complex network of inductive and inhibitory signals within the heart and from

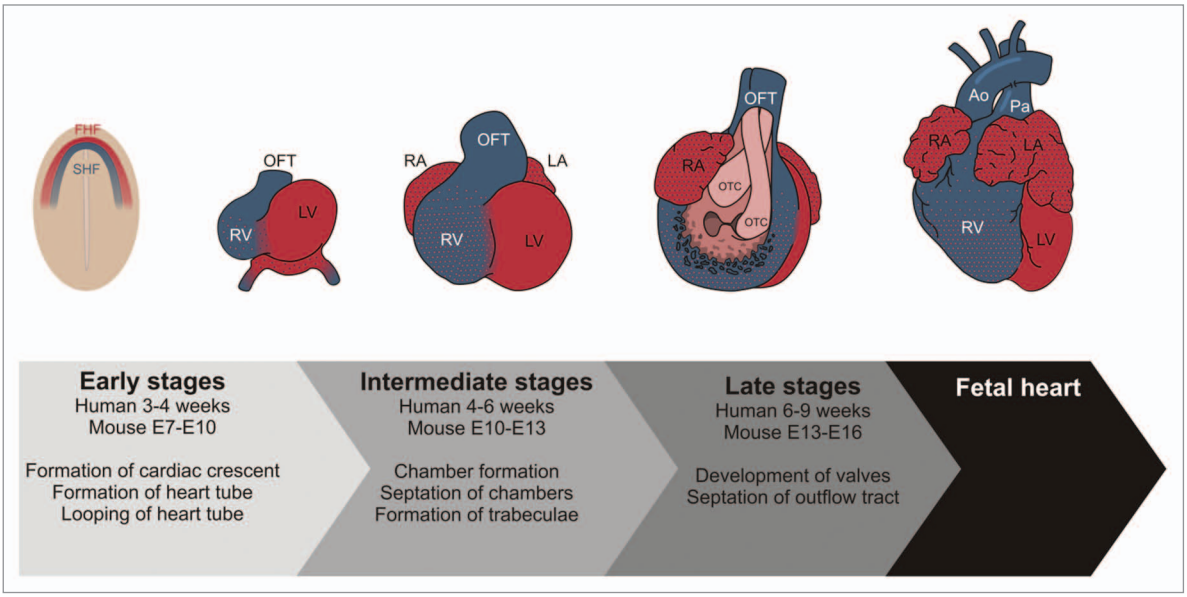

Figure 2. Overview on developmental stages of the heart. The cardiac crescent is formed around day 15 in humans. Myocardial progenitors from the first heart field (FHF, red color) contribute to the ventricles, the atria and the atrioventricular canal (AVC). Progenitors from the second heart field (SHF, blue color) contribute to the outflow tract (OFT) and all other regions of the heart, except the left ventricle. A linear heart tube is formed around day 21 in humans. The heart tube loops and chambers are formed by ballooning of regions destined to become atrium and ventricles. Endocardial cushions form in the AVC and the OFT. These cushions will later transform into the semilunar and atrioventricular valves and will participate in the septation of the OFT. Abbreviations: Ao, Aorta; LA, left atrium; LV, left ventricle; OTC, outflow tract cushions; Pa, pulmonary artery; RA, right atrium; RV, right ventricle.

surrounding tissues that impact on cardiac specification, differentiation, and maturation. Initially, a series of craniolateral endoderm-derived inductive signals combined with inhibitory signals derived from midline structures regulate early cardiogenesis.[82] In all cases, the signals are controlled and interpreted by multiple signaling pathways, of which many are involved in coordination of several stages of heart development. As an example, the Hh signaling pathway plays an important role in establishing L-R asymmetry in vertebrates.[83-85] In canonical Hh signaling, Hh ligands bind to and inhibit the activity of the transmembrane receptor Patched (Ptch), allowing another transmembrane receptor, Smoothened (Smo) to promote the activation of Gli transcription factors that control cellular processes during development and in tissue homeostasis.[86] The heart is the first organ to break the embryonic bilateral symmetry, and the asymmetric looping of the heart tube is important for correct chamber development and septation.[11] Targeted deletion of the Hh ligand gene, *Sonic hedgehog (Shh)*, results in atrial and ventricular septum defects and abnormal development of the OFT in mice.[87] In agreement with this, atrial septum progenitor cells and cells of the pulmonary trunk show Hh responsiveness in mouse embryos.[88] Similarly, inhibition of Hh signaling in chicken embryos results in pulmonary atresia and stenosis as well as persistant truncus arteriosus (PTA).[89] In chicken embryos, *SHH* is expressed in the pharyngeal endoderm, adjacent to the SHF, where *PTCH* is expressed, suggesting a requirement for Hh signaling in SHF.[89] In line with this, tissue-specific deletion of Hh signaling components suggests that Hh signaling is required within the SHF and cardiac neural crest for normal development of the OFT[87,90] and within the dorsal mesocardium for atrioventricular (AV) septation and AV valve development.[91]

The TGFβ/Bone Morphogenic Protein (BMP) signaling network also plays a critical role during heart development.[92] This network comprises a multitude of different ligand types that act through the activation of a family of transmembrane receptor serine/threonine kinases, which in part activate Smad transcription factors to elicit different cellular responses during development and in tissue homeostasis.[92,93] Nodal, Lefty1, and Lefty2 are all members of the TGFβ ligand superfamily. Like Shh, Nodal and Lefty1/2 are asymmetrically expressed in the node and involved in establishment of L-R asymmetry in the mouse embryo (see above, reviewed in ref. 10). In line with this, mutations in the *NODAL* and *LEFTY2* genes cause heterotaxy and/or isolated CHD in humans.[94,95] In vitro collagen gel experiments, using AV or OFT explants, suggest that TGFβ signaling is involved in EMT and EndoMT during the formation of endocardial cushions,[96-98] and targeted mutation of genes encoding TGFβ ligands causes defects of the OFT (double outlet right ventricle (DORV)), abnormal semilunar valves, and AV cushions, supporting an involvement in development of the endocardial cushions.[99,100] Null mutants for the TGFβ receptor genes, *Tgfbr1* and *Tgfbr2*, are embryonic lethal in mice, but tissue specific deletion of TGFβ receptor genes support a role for TGFβ signaling in development of the OFT.[101,102] Targeted deletion of *Tgfbr1* or *Tgfbr2* in neural crest cells leads to OFT defects (PTA, interrupted aortic arch and ventricular septal defect [VSD]), strongly suggesting that TGFβ signaling is necessary for cardiac neural crest cells to promote normal septation of the OFT.[101-103] Other mouse models with targeted deletions in BMP/TGFβ signaling

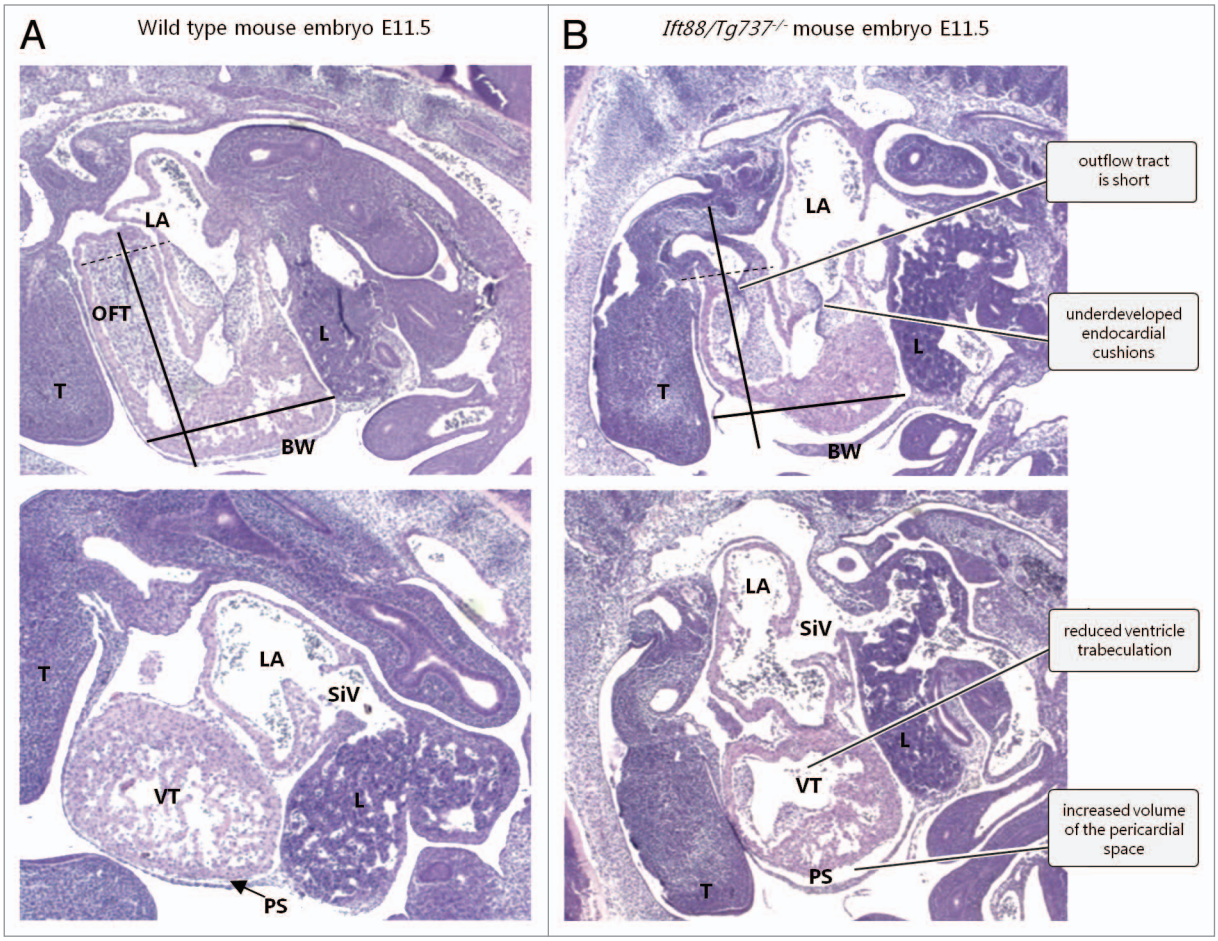

Figure 3. Defects in ciliogenesis lead to CHD in *Ift88/Tg737*$^{-/-}$ mouse embryos (E11.5). Upper panels: longitudinal mid-sagittal sections. Lower panels: comparable longitudinal para-sagittal sections. Abbreviations: BW, body wall; L, liver; LA, left atrium; OFT, outflow tract; PS, pericardial space (arrow); SiV, sinus venosus; T, tongue; VT, ventricle. The black bars have identical dimensions in wt (**A**) and mutant (**B**) embryos. The distal end of the OFT is marked with a dotted line. The figure is modified from reference 21 with permission.

component genes support a role of this signaling network in heart development. Deletion of *Smad6*, which inhibits TGFβ/BMP signaling, causes valvular hyperplasia and OFT defects in mice.[104] Further, deletion of *Ltbp1*, encoding latent TGFβ-binding protein 1, causes OFT defects,[105] whereas cardiac specific deletion of *Bmp2* causes AV cushion defects,[106] and cardiac specific deletion of *Bmp4* results in OFT defects.[107] In humans, genomic deletion of *TGFBR2*[17] and point mutations in *SMAD6*[108] and *SMAD2*[109] have been associated with heart defects in the form of heterotaxy (*TGFBR2, SMAD2*) and valve defects (*SMAD6*).

Nodal Cilia and Heterotaxy

During gastrulation, both motile and immotile (primary) cilia are thought to play a critical role for the establishment of the L-R asymmetry of the body and proper placement and patterning of the internal organs and associated vasculature, including looping of the heart.[6,39] Within the node, which is formed after definition of the dorsoventral and anteroposterior axes,[110,111] motile nodal cilia (**Fig. 1A**) rotate synchronously to produce a leftward movement of embryonic fluid, i.e. the nodal flow. The correct positioning of nodal cilia is a prerequisite for the nodal flow, and a process that relies on planar cell polarity (PCP) signaling (reviewed in ref. 39). The nodal flow is essential for defining lateral asymmetry[8,67,112,113] in part through activation of the Nodal signaling cascade. Initially, the gene encoding actin binding lim protein 1 (*Ablim1*) is evenly expressed across the node but Ablim1 mRNA gradually disappears from the left side of the node in response to nodal flow, at least partially independent of the Nodal signaling cascade.[114] Simultaneously, Nodal accumulates at the left side of the embryo, conferring left-side specific *Pitx2* expression and asymmetric morphogenesis.[115,116]

A total of 200–300 cilia generate the nodal flow in the mouse, but it has been suggested that as little as two motile cilia at the node can break the bilateral symmetry in the mouse embryo.[111] Several models have been introduced to explain how nodal flow regulates Nodal signaling and establishment of L-R asymmetry (reviewed in ref. 6). Two models suggest that nodal flow creates an asymmetric distribution of morphogens across the node resulting in accumulation of signaling molecules at the left side of the node — either as soluble molecules in the embryonic fluid or as encapsulated molecules that are delivered to the left side of the

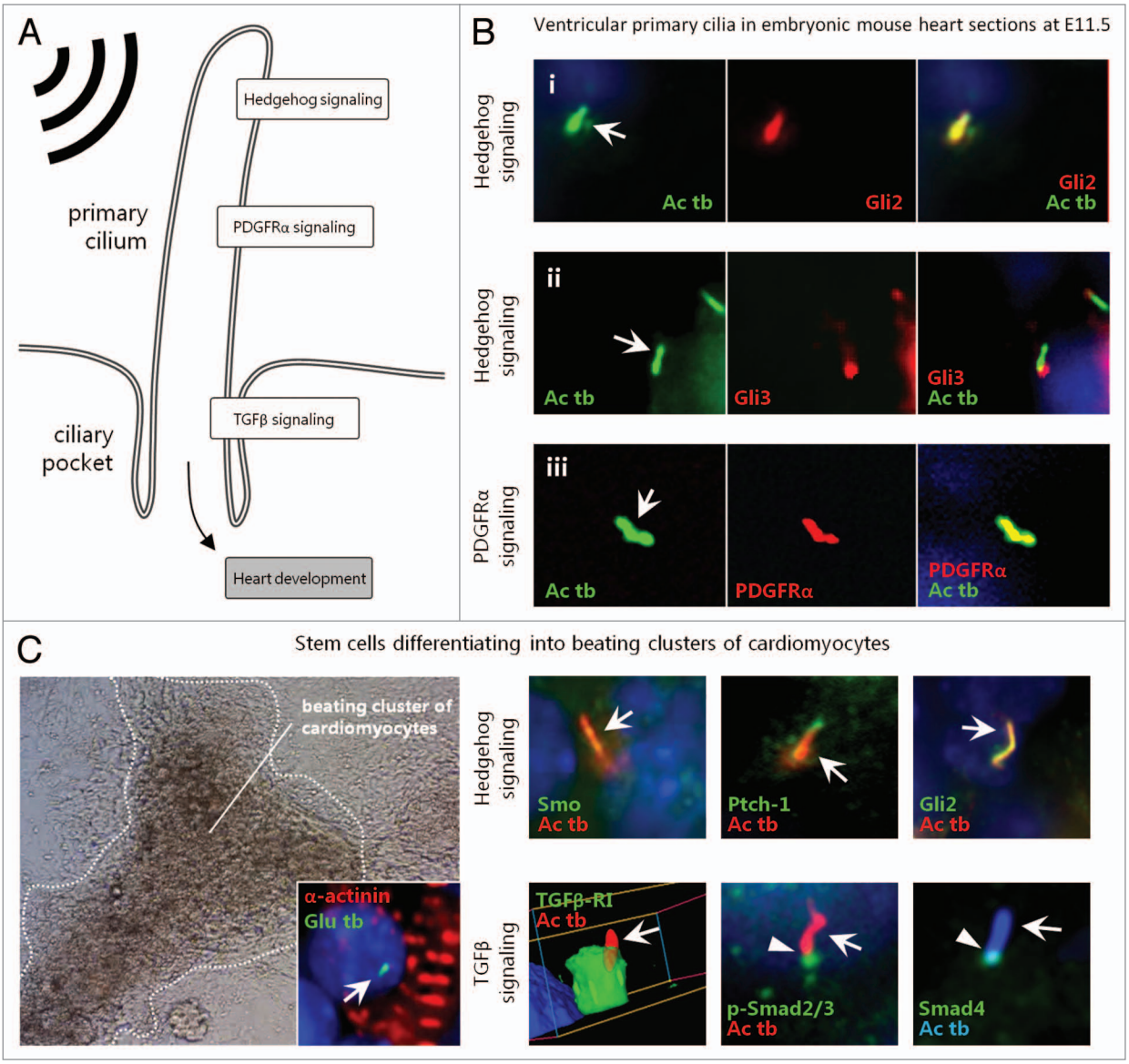

Figure 4. Primary cilia and signaling pathways in cardiomyogenesis and heart development. (**A**) Schematic drawing of signaling pathways in the cilium. (**B**) Immunofluorescence microscopy (IFM) analysis of Hedgehog (Hh) and Platelet-Derived Growth Factor Receptor α (PDGFRα) signaling components in ventricular primary cilia in transverse embryonic mouse heart sections at E11.5. Primary cilia (arrows) were marked with an antibody against acetylated α-tubulin (Ac tb; green). Signaling components (red) were marked with antibodies against transcription factors in Hh signaling (Gli2 and Gli3; upper and middle row images) and PDGFRα (lower row images). Nuclei were marked with DAPI, which stains DNA (blue). Reproduced from reference 21 (**i**) and 22 (**ii and iii**) with permissions. (**C**) Left image: Light microscopy analysis of a beating cluster of cardiomyocytes differentiated from mouse embryonic stem cells and IFM image (inset) of a primary cilium (arrow) marked with glutamylated α-tubulin (Glu tb; green) in cardiomyocytes expressing α-actinin (red). Nuclei were marked with DAPI (blue). Right images: IFM analysis on the localization of Hedgehog and Transforming Growth Factor β (TGFβ) signaling components to primary cilia (Ac tb, open arrows) in stem cells undergoing cardiomyogenesis. The ciliary pocket area is indicated with arrow heads. Hh signaling components (Smoothened [Smo], Patched-1 [Ptch-1] and Gli2, all green) localizes along the entire length of the cilium (*red*) - nuclei were marked with DAPI (blue) (upper row images). TGFβ signaling components (TGFβ Receptor I [TGFβ-RI], phospho-Smad2/3 [pSmad2/3] and Smad4, all green) localize around the ciliary pocket area (lower row images). For Smad4 localization, the cilium is indicated with blue fluorescence. Reproduced from references 20 and 21 with permissions.

node in membrane-bound vesicles, also known as nodal vesicular parcels (NVPs) (reviewed in ref. 39). In the latter scenario, NVPs contain Shh and retinoic acid that induce an increase in the level of intracellular Ca^{2+} on the left side of the node and subsequent generation of L-R asymmetry.[117] A third model suggests that the nodal flow initiates a Ca^{2+} influx in crown cells on the left side of the node, via activation of the polycystins (PC2 and the PC1 homolog, PC1 like 1) in mechanosensory, immotile primary cilia.[7,9,118,119] In support of the latter model, the asymmetric expression of *Ablim1* at the node during the mid-headfold stage requires both a nodal flow and PC2, but the details of this relationship have not been resolved.[114] Further, it was recently shown

that glycosylation of the Notch receptor in the crown cells by the N-acetylgalactosamine-type O-glycosylation enzyme GALNT11 is essential for the optimal ratio between motile and immotile cilia at the mouse node, in addition to correct *Pitx2* expression in *Xenopus* embryos.[120] Importantly, however, the described models are not mutually exclusive, and since the breaking of bilateral symmetry is a crucial step in embryonic development, it is possible that several mechanisms exist to ascertain this process, up- and downstream of the nodal flow.[114,121]

The importance of nodal cilia in establishing L-R asymmetry was first revealed by the laboratories of Martina Brueckner and Nobutaka Hirokawa. Mouse embryos with deletion in either *Kif3a* or *Kif3b* displayed absence of nodal cilia with accompanied loss of nodal flow and L-R abnormalities, including defective cardiac looping,[8,122] and mutation in *Lrd*, disrupting ciliary motility, produced a similar phenotype.[113,123] Since these initial discoveries, several additional genes and gene products that regulate ciliary formation, maintenance, and function have been found to be critical for generating L-R asymmetry. These include genes encoding transcription factors such as *Tbx6, Rfx3, Hfh-4*, and *ZIC3*, which when defective lead to defects in formation of nodal cilia as well as situs abnormalities including *situs inversus* (SI) and heterotaxy.[16,124-126] Further, multiple genes important for ciliary positioning, motility and disassembly have been associated with situs abnormalities and heterotaxy, often associated with cardiac defects such as DORV, septal defects, transposition of the great arteries (TGA), TOF, single ventricle (SV), and coarctation of the aorta (CoA) in mice and/or zebrafish (reviewed in refs. 65,127,128). As an example, ciliary NPHP2/inversin is required for the generation of a proper nodal flow,[67] and potentially controls nodal cilia positioning.[39]

In human studies, patients with mutations in genes required for ODA assembly and function often have motility-defective cilia and situs abnormalities.[128,129] In a cohort of patients with ciliary motility defects, the incidence of heterotaxy was highly increased (6.3%) compared with the general population (0.004%). Further, the incidence of CHD is significantly increased in heterotaxy patients, when compared with the general population (57% vs. 1% respectively)[129,130] with septal defects and TGA being the most prevalent CHDs.[129] In an ENU mutagenesis screen in mice,[131] a mutation in the gene encoding Dnah5 (a dynein heavy chain) was found to cause situs abnormalities, including heterotaxy (40%) and SI (35%), and many of the mice had associated CHD similar to those observed in the study by Kennedy and colleagues.[129] Together, these studies reflect the importance of nodal cilia and their motility in situs establishment.

In summary, mutations affecting the structure and function of nodal cilia lead to L-R asymmetry defects associated with CHD. However, it is important to realize that such mutations may also impact on the assembly and/or function of primary cilia formed within the developing heart, making it difficult to distinguish between the function of different populations of cilia in time and space during heart development; for example whether a given heart defect results from defective nodal and/or cardiac primary cilia. Nevertheless, as will be discussed below, multiple lines of evidence indicate that a series of cilia-based heart defects may be associated specifically with defects in cardiac primary cilia.

Cardiac Primary Cilia in Heart Development

Cardiac primary cilia were first discovered in 1969 when Rash and colleagues identified primary cilia throughout the embryonic and adult heart in chickens, mice, rabbits, and lizards.[132] Later, cardiac primary cilia were also observed in the embryonic and adult human heart,[133] as well as other parts of the cardiovascular system, including endothelial cells of the aorta[134] and cultured human umbilical vein endothelial cells.[135] The distribution of cardiac primary cilia during cardiogenesis is poorly resolved; however, a few papers have described a spatiotemporal distribution during heart development and morphogenesis. In E9.5 mouse embryos, where the early heart tube has looped and is contracting, primary cilia are lining the endothelium of both atria primordia, the forming ventricular trabeculations and the endothelium lining the developing endocardial cushions.[136] At E12.5, primary cilia are also found in the epicardium and in the mesenchymal cells of the endocardial cushions. The ventricular trabeculations remain ciliated, but the atria primordia are now less ciliated and the endothelium lining the endocardial cushion is de-ciliated.[136] For examples of cardiac primary cilia, please see **Figure 1B**. The re-absorption of cilia in the endothelium of the endocardial cushions is in agreement with other studies showing that primary cilia are resorbed in response to sheer stress.[135,137-139] Fluid shear stress is known to play important roles in trabeculation, cardiomyocyte proliferation, and establishment of the conduction system, and changes in the fluid flow leads to CHD.[140] Interestingly, mouse endothelial cells that form stunted primary cilia (e.g derived from the *Tg737*^{orpk} mutant*)* or are mutated in *Pkd1* show impaired response to shear stress,[141] and mice with mutations in *Kif3a, Lrd*, *Pkd2,*[136] and *Ift88/Tg737*[21,142] have defects in chamber maturation.[21,136,142]

A variety of different vesicle trafficking pathways regulate the assembly, maintenance, and sensory function of cilia.[143,144] Often, the proximal part of the cilium is placed within an invagination of the plasma membrane known as the ciliary pocket, which functions as the ciliary platform for exocytosis of Golgi-derived vesicles and for clathrin-dependent endocytosis (CDE).[145] Available evidence suggests that exocytosis is required for the targeting of transmembrane receptors and ion channels to the ciliary membrane, and CDE may regulate ciliary signaling through the internalization and/or recycling of receptors at the ciliary base.[20,146] In support of a critical role of cilia in heart development, mutations in genes involved in trafficking of vesicles to and from the cilium are associated with CHD. Mutant mouse embryos lacking the Golgi-associated protein, Gmap210, which is required for Ift20-mediated trafficking and localization of PC2 to the cilium,[147] die around the time of birth and show heart defects including VSD and TOF.[147] In agreement with this, *Pkd2*$^{-/-}$ mouse embryos display heart defects reminiscent of those observed in *Gmap210*$^{-/-}$ mouse embryos.[148] Further, *Pifo*$^{-/-}$ E7.5 mouse embryos, which lack Pitchfork that interacts with components of the endocytic machinery, including the small GTPases

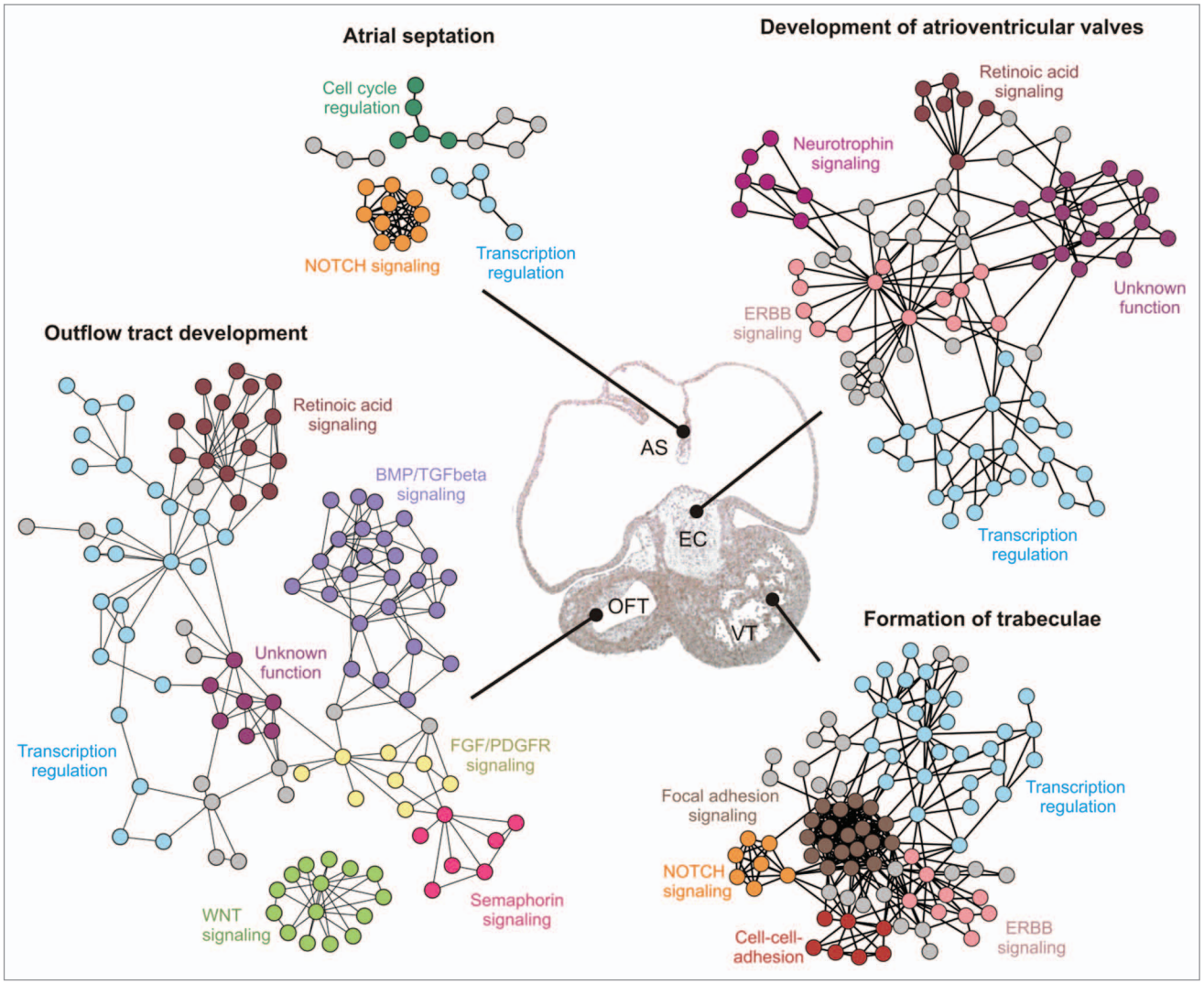

Figure 5. Examples of signaling networks involved in development of specific anatomical structures of the heart. Protein-protein interaction networks involved in atrial septation, outflow tract development, atrioventricular valve development and formation of trabeculae are shown. Functional clusters within the networks are color coded. Tissues affected by the networks are marked in a hematoxylin-eosin stained frontal section of the heart from a 37 d human embryo. Abbreviations: AS, atrial septum; EC, endocardial cushions; OFT, outflow tract; VT, ventricle. The figure is modified from reference 212.

Rab8, Rab11 and Arl13b, and induces the resorption of the primary cilium before entry into mitosis,[19] display double-ciliated cells in the nodal pit as well as L-R defects with associated cardiac defects, including DORV and right ventricle hypoplasia.[19] Other proteins that are important for intracellular trafficking pathways in relation to cilia and have been associated with CHD include the Alström syndrome protein 1, ALSM1, and the sorting Nexin, SNX10.[149,150] ALMS1 localizes to the pericentriolar region together with Rab11 and early endosomes, and in isolated fibroblasts from ALMS patients the kinetics of transferrin uptake by CDE is increased compared with that in wild type (wt) fibroblasts.[149] SNX10 affects the ciliary localization of Rab8a, and in *snx10* mutant zebrafish, cardiac looping is affected.[150] In support of a role for nexins in cardiogenesis, a patient with TOF was recently found to have a rare genomic deletion which include the *SNX8* gene.[130] In relation to the different functions of nodal and cardiac primary cilia at different time points during development, it is likely that some of the heart defects found in ciliary mutants may be caused by events later in heart development, and could thus be triggered by defects in cardiac primary cilia.[21,136]

Mutations in other genes involved in ciliogenesis also causes CHD. *Ift88/Tg737*$^{-/-}$ mouse embryos with stunted cilia develop severe CHD including atrial septal defects (ASD), VSD, atrioventricular septal defect (AVSD), and OFT septal defects[21,136,142]; defects that are all associated with malformed endocardial cushions (**Fig. 3**). Development of the endocardial cushions depends on EndoMT, a cellular process whereby endothelial cells lose the epithelial phenotype and differentiate into mesenchymal cells that ingress into the underlying cardiac jelly.[151] The endocardial cushions in *Kif3a*$^{-/-}$ and *Pkd2*$^{-/-}$ mouse embryos are acellular and embryos with mutation in the *Ift88/Tg737* gene have a decreased amount of mesenchymal cells in the endocardial cushions. Furthermore, endocardial cells derived from *Ift88/Tg737*$^{-/-}$ mice show disturbances in EndoMT.[136,142,151] Taken together, this

suggests that cardiac primary cilia are involved in formation and development of the endocardial cushions. Indeed, the regulation of signaling networks during heart development has in some cases been linked to the function of cardiac primary cilia,[20-22] indicating the complexity by which different populations of cilia may contribute to the formation the heart in a spatiotemporal manner. However, we still know little as to how cardiac primary cilia contribute to heart development independently of nodal cilia and downstream of situs establishment, and whether CHD may arise exclusively by defects in the sensory function of primary cilia in the tissues of the developing heart.

Cardiac Primary Cilia and Coordination of Hedgehog Signaling

Hh signaling is coordinated by primary cilia in a variety of different cell types to regulate tissue patterning and homeostasis in vertebrates (reviewed in refs. 29, 86, 152, and 153). The cilium functions as a unique compartment for the continuous turnover of Hh signaling components, such as Ptch-1, Smo, and Gli transcription factors and other regulatory proteins to control cellular processes. Smo becomes localized to the primary cilium through the binding of Hh ligands to Ptch-1, which then induces Smo-dependent activation of Gli transcription factors in the cilium. Consequently, defects in ciliary assembly or turnover of Hh components in the cilium lead to a plethora of developmental disorders and diseases, including CHD.[21,22,136,142]

A number of observations have specifically linked cardiac primary cilia to the regulation of Hh signaling. Components in this pathway localize to primary cilia in embryonic hearts as well as in cardiomyocytes differentiating from stem cells in vitro (**Fig. 4**),[21,22,142] and defective ciliary assembly is associated with defective cardiomyogenesis[21] as well as Hh-related heart defects that might be independent of nodal cilia, including ventricular and endocardial cushion-derived defects.[122,142,154] Further, 60% of patients with mutations in genes encoding the EVC proteins, EVC1/EVC2, which interact with Smo at the primary cilium to transduce Hh signaling[155-159], display CHD, including AVSD and ASD.[47,51] In line with these findings, EVC proteins are co-expressed in the OFT and the mesenchymal structures of the atrial septa and AV cushions during heart development in mice[51]; areas that are known to be ciliated during cardiogenesis.[136,142]

Septation of the ventricles is also dependent on the gene *Fantom* (*Ftm*)[22] that encodes the ciliary base protein Rpgrip1l/Nphp8, which is required for proper ciliogenesis, PCP, and Shh signaling, and when mutated leads to a series of ciliopathies.[22,52-54,160-165] In *Ftm* null mouse embryos, 33% of all analyzed embryos had VSD in the membranous part of the septum, 81.5% had defects in the muscular part, and all analyzed embryos had reduced atrial and ventricular wall thickness.[22] In the ciliated areas of $Ftm^{-/-}$ embryonic hearts at E11.5, the rate of proliferation and Shh signaling was markedly reduced. In contrast, no change was observed in non-ciliated areas,[22] suggesting that Ftm relies on cardiac primary cilia for signaling, and that the heart phenotypes in the $Ftm^{-/-}$ mouse are primary cilia-dependent. Ftm seems to be involved in the processing of Gli3,[161] and in agreement with this, the amount of unprocessed Gli3 is 10-fold higher in $Ftm^{-/-}$ hearts compared with heart of control littermates.[22] The cardiac phenotypes described above suggest that Hh signaling is important for proliferation and differentiation of cardiomyocytes and are thus in agreement with the previous findings that primary cilia in part coordinate Hh signaling during the differentiation of stem cells into cardiomyocytes.[21,166] Finally, defects in proper trafficking of Smo and Ptch1 in and out of the cilium due to mutations in *Ift25* lead to cardiac phenotypes in mice reminiscent of those found in Hh signaling mutants, including defects involving the ventricle, OFT and AVC.[154]

Cardiac Primary Cilia and Coordination of TGFβ Signaling

The superfamily of TGFβ/BMP signaling pathways is involved in a vast majority of cellular processes and is therefore fundamentally important during development and in tissue homeostasis.[92] The functional output of these pathways highly relies on their extensive cross-talking with other receptor-mediated signaling systems, leading to synergistic or antagonistic effects on tissue patterning and organ function.[92] Consequently, deregulation of TGFβ/BMP activity almost invariably leads to pathologies in the adult as well as severe developmental defects, including malformation of the OFT, AVC and septa.[19,167] Interestingly, defects in these areas are also found in cilia-related mutants. Further, genomic deletion and duplication of the human gene encoding TGFβ Receptor II, *TGFBR2,* causes heterotaxy,[17] whereas endothelial cells derived from $Ift88/Tg737^{-/-}$ mouse embryos show defective TGFβ signaling upon sheer induced EndoMT.[151] These results indicate that TGFβ signaling is important at multiple stages during heart development, i.e., both up- and downstream of L-R determination.

We recently showed a function of the primary cilium in regulating canonical TGFβ signaling through the activation of Smad2/3 transcription factors at the ciliary pocket.[20] This signaling pathway is regulated by CDE where internalization of ligand-bound TGFβ receptors in clathrin coated vesicles (CCVs) and early endosomes (EEs) allows Smad2/3 to become activated by receptor-mediated phosphorylation.[168,169] In several cases, cardiac primary cilia emerge from a ciliary pocket[133,142] and during the differentiation of murine carcinoma stem cells as well as human embryonic stem cells (hESC) into cardiomyocytes, TGFβ receptors and activated Smad2/3 accumulate at the ciliary base (**Fig. 4**).[20] Further, the TGFβ ligand, TGF-β1, stimulates the differentiation of stem cells into cardiomyocytes, and mouse embryonic fibroblasts derived from mice with a hypomorphic mutation in *Ift88/Tg737* ($Tg737^{orpk}$) show decreased TGFβ signaling associated with reduced CDE activity at the ciliary base.[20] These findings support the conclusion that cardiac primary cilia play a direct role in coordinating TGFβ signaling during cardiomyogenesis. It remains to be investigated whether BMP signaling also is associated with the primary cilium during heart development.

Cardiac Primary Cilia and Coordination of PDGF Signaling

PDGF signaling is mediated by a series of ligands (PDGF-AA, -AB, -BB, -CC, and -DD), which bind to and activate either homo- or hetero-dimers of the RTKs, Platelet Derived Growth Factor Receptor α (PDGFRα) and Platelet Derived Growth Factor Receptor β (PDGFRβ).[170] Previous studies showed that PDGFRα specifically localizes to primary cilia in a number of cell types and tissues, including the heart,[22,171-176] and that activation of the receptor and its downstream effectors, Mek1/2-Erk1/2-Rsk and Pi3K-Akt, is initiated in the cilium to regulate cell cycle control and directional cell migration in fibroblasts.[171,177,178]

The recent finding that PDGFRα localizes to primary cilia in E11.5 mouse heart ventricles[22] (**Fig. 4**), indicates that part of the PDGF signaling network is associated with cardiac primary cilia during heart development. Ciliary localization of PDGFRα is downregulated in both Ftm- and Shh-negative ventricles, suggesting that PDGFRα signaling acts downstream of Hh signaling in cardiac primary cilia and that defects in these cilia are associated with reduced ventricular cell proliferation leading to diminished ventricular wall thickness and VSD.[22] Interestingly, PDGFRα and its specific ligands, PDGF-A/C,[179] localize to the ventricles and the OFT and AV cushions, and the myocardium of the OFT, VS, and AS during heart development.[180,181] These regions correspond to ciliated areas of the heart, further emphasizing a link between PDGFRα signaling and the primary cilium. In particular, the spatiotemporal expression of PDGFRα and PDGF-A/C ligands suggests a role of this signaling pathway during remodeling of the myocardium and in septation of atria, ventricles, and OFT,[180,182,183] and in agreement with this, alteration in PDGF signaling affects myofibril differentiation and migration.[184,185] Furthermore, studies from mice show that mutated or absent PDGFRα, results in prenatal death and heart defects including thinned myocardium, and septa, valve, OFT, and aortic arch malformations.[186-188] In conclusion, part of the PDGF signaling system might be specifically coordinated by cardiac primary cilia, potentially in a network with Hh and other signaling pathways to coordinate cardiogenesis.

Cardiac Primary Cilia and Other Signaling Pathways

In addition to Hh, TGFβ, and PDGFRα signaling, multiple other signaling networks critically regulate heart development, including Wnt and Notch signaling.[82,189-194] The Wnt signaling network consists of a highly complex signaling arrangement that traditionally is divided into canonical and non-canonical Wnt signaling.[195] In canonical signaling, the binding of Wnt-ligands to Frizzled (Fz) receptors facilitates stabilization of β-catenin, which mediates transcription of canonical Wnt target genes in cell proliferation and differentiation. Non-canonical Wnt signaling operates independently of β-catenin via Dishevelled (Dvl) and its various interaction partners, such as Vangl2 and Celsr, resulting in polarization of cells and tissues, e.g. PCP, to promote cell migration and convergent extension movements.[2,189,192,193,196] The link between Wnt signaling and the primary cilium is highly controversial.[32,35,197] However, many Wnt signaling components, such as Fz3, Dvl, and β-catenin, localize to the ciliary/centrosomal axis, and dysregulated Wnt signaling has been reported in cells and tissues with disrupted ciliogenesis or basal body integrity.[198-200] Moreover, in the mouse inner ear, establishment of PCP was shown to depend on the formation of functional primary cilia.[201] However, other studies in mice and zebrafish reveal no obvious canonical or non-canonical Wnt phenotypes in cilia mutants,[202,203] emphasizing the importance of further studies in this area.

Multiple PCP components are strongly expressed in the OFT during early heart development, and when defective, result in OFT and ventricular defects.[2,189] Mutations in *Vangl2* disrupt migration of cells into the OFT and this is associated with impaired OFT myocardialization as well as ventricular and OFT septal defects.[189] The localization of Vangl2 during heart development is dependent on Scribble, another PCP component,[204] and in agreement with this, *Scribble* phenotypes are reminiscent of *Vangl2* mutants.[204,205] In support of the idea that non-canonical Wnt signaling is associated with cardiac primary cilia, *Gmap210*$^{-/-}$ mouse embryos with impaired ciliogenesis due to impaired ciliary targeting have cardiac phenotypes resembling those observed in multiple PCP mutants, including *Vangl2*, *Dvl2,* and *Scribble* mutant mice.[147] Although kidney-specific depletion of Ift20 in mice resulted in defective Wnt signaling in the affected tissues,[206] it remains to be determined whether the cardiac phenotypes observed in the *Gmap210*$^{-/-}$ mouse are directly associated with defective non-canonical Wnt signaling. Interestingly, NPHP2–4 and Ftm/Rpgrip1l/NPHP8 all seem to impair canonical Wnt-signaling while promoting non-canonical Wnt responses.[14,165,200,207-209] As an example, NPHP2/inversin and the structurally related diversin in zebrafish are required for convergence extension movement in vertebrates, and depletion of either result in cardiac defects.[190,208] Together, these aspects suggest that inversin and other NPHPs are implicated in heart development by controlling Wnt signaling, but whether this is coordinated by cardiac primary cilia remains speculative at this point.

During cardiogenesis, Notch signaling is important for development of the OFT and ventricular trabeculation.[194] Cell-cell contact is required for Notch signaling as the ligands for the Notch receptors (1–4) are transmembrane proteins and include proteins from the Delta and Jagged family. Ligand binding to the Notch receptors induces cleavage and release of NICD, the intracellular part of the receptor that translocates to the nucleus to regulate gene transcription.[194,210] Recently, Notch signaling was associated with the primary cilium during skin development, as Notch3 and presenilin-2 localize to the primary cilium in suprabasal epidermal cells, and defective IFT results in altered Notch signaling.[31] The expression of Notch components correlates with ciliated areas of the heart during development, and defective Notch signaling leads to cardiac phenotypes reminiscent of cilia-related phenotypes.[194,210,211] It was recently shown that glycosylation of the Notch receptor is important for specifying non-motile primary cilia in the frog gastrocoel roof plate (frog node),[120] although a direct link between Notch signaling

and primary cilia in cardiomyogenesis and heart development remains to be proven.

Concluding Remarks and Perspectives

Primary and motile cilia are required throughout heart development. Initially, at E8 in the mouse, non-motile sensory cilia on crown cells at the periphery of the node and motile cilia at the center of the node are required for generating the L-R asymmetry of the embryo and correct looping of the heart. At later stages, primary cilia in the developing heart may coordinate signaling pathways important for organizing morphogenesis and maturation of the heart. Consequently, defects in assembly or function of nodal and cardiac primary cilia may lead to CHD.

Solexa-based transcriptomics of murine stem cells differentiating into beating clusters of cardiomyocytes showed that this process involves time-dependent differential expression of genes within the Mitogen Activated Protein Kinase (MAPK), Wnt, TGFβ/BMP, Hh, Epidermal Growth Factor Receptor (EGFR) and Notch signaling pathways,[20] and both Hh and TGFβ signaling are associated with primary cilia forming within these clusters of cardiomyocytes.[20,21] Lage et al. (2010) performed a genome-wide systematic mapping of protein-protein interaction networks involved in different stages of heart development based on mouse models.[212] In this study, high-confidence experimental interactome data suggested that heart development is controlled by communication within and between a defined set of signaling pathways (**Fig. 5**). As such, these signaling pathways seem to function as recycled signaling modules, which integrate into higher-order networks to control the different stages of heart development. Keeping in mind that such an analysis only covers part of the signaling networks involved in heart development, we note that many of these cross-talking functional modules include signaling pathways known to be coordinated by the primary cilium. These signaling modules include TGFβ/BMP signaling, Fibroblast Growth Factor Receptor (FGFR) signaling, PDGFR signaling, Notch signaling, EGFR signaling and Wnt signaling among others.

Because so many different signaling systems are associated with primary cilia,[20,21,31,171,172,175,200,213-216] we suggest that primary cilia may function as signaling hubs in the spatiotemporal cross-talking between diverse signaling networks during heart development. Future work should therefore focus on how primary cilia are involved in the integration and cross-talking between different signaling networks and how this may impact on different stages of heart development. Here, it will be important to differentiate between signaling involving cilia in the node and signaling involving primary cilia in cardiac tissues at later developmental stages. Thus, it will be important to include analysis with conditional knockout of primary cilia from various heart tissues at different time points and investigate how cardiac primary cilia function in the progressive differentiation, morphogenesis and maturation of the heart independent of nodal cilia and early L-R specification.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank all authors who have given permission for their micrographs to be used in the article. We apologize to those investigators whose work has not been cited due to space limitations. This work was supported by grants from the Lundbeck Foundation, The Novo Nordisk Foundation, The Danish Council for Independent Research, Nordforsk, The Danish Cancer Society, The Danish Heart Association and the UCPH Excellence Programme for Interdisciplinary Research (2016 Funds), University of Copenhagen, Denmark.

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